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# The Glucagon-sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver

## I. PROPERTIES

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### SUMMARY

**The liver parenchymal cell plasma membrane preparation devised by Neville (*Biochem. Biophys. Acta*, 154, 540 (1968)) contains an adenyl cyclase system which is stimulated by glucagon and to a much lesser extent by epinephrine. The yield of adenyl cyclase can be greatly increased, at the expense of some contamination by other organelles, by increasing the amount of starting material and eliminating the last step in the preparative procedure. Adenyl cyclase activity is 25-fold purified in the membranes compared to a liver homogenate.**

**Liver membrane adenyl cyclase activity is a function of glucagon concentration over a range of $10^{-10}$ to $10^{-7}$ M glucagon and is increased more than 10-fold by a maximally stimulating concentration of glucagon. The stimulation produced by epinephrine is less than 10% of that produced by glucagon. Secretin, a polypeptide hormone very similar to glucagon in its primary structure, does not stimulate liver membrane adenyl cyclase activity. Insulin changes neither the basal nor the glucagon-stimulated adenyl cyclase activity.**

**Adenyl cyclase activities, under standard assay conditions, are proportional to time of incubation and to membrane concentration. The enzyme requires a divalent cation, either $Mg^{++}$ or $Mn^{++}$, but is inhibited by $Ca^{++}$. At sufficiently high concentrations, both ATP and $Mg^{++}$ inhibit the enzyme. Addition of 1 mM EDTA to the assay medium doubles the glucagon-stimulated activity. Fluoride ion stimulates adenyl cyclase activity but to a lesser extent than does glucagon. Glutaraldehyde, *N*-ethylmaleimide, and *p*-chloromercuribenzoate inhibit the adenyl cyclase system. Urea, at a concentration of 2 M, reduces both the maximal glucagon- and fluoride-stimulated activities, but at 0.4 M reduces only the apparent affinity of the system for glucagon.**

**Attempts to solubilize and purify the adenyl cyclase activity in an active form from rat liver membranes have been unsuccessful.**

Adenyl cyclase in mammalian tissues is a membrane-bound enzyme system which catalyzes the reaction (1, 2)

$$ATP \rightarrow \text{cyclic AMP}^1 + PP_i$$

Certain polypeptide hormones and catecholamines exert at least part of their effects by stimulating the activity of this enzyme in their target cells (3). However, the mechanism, in molecular terms, by which these hormones stimulate adenyl cyclase activity is poorly understood.

In a previous communication (4) we reported the presence of a glucagon-sensitive adenyl cyclase system in parenchymal cell plasma membranes prepared from rat liver by the procedure of Neville (5). We have subsequently found that the yield of adenyl cyclase can be greatly increased, at the expense of some contamination by other organelles, by omitting part of the Neville procedure. This modified preparation has several desirable features as a starting material for studies of the mechanism of hormonal stimulation of adenyl cyclase. The purpose of this paper, the first in a series (6–9), is to describe pertinent general features of the liver membrane preparation and its adenyl cyclase system.

### EXPERIMENTAL PROCEDURE

#### *Materials*

Glucagon (crystalline) was supplied by Lilly. Secretin was a gift from Dr. Victor Mutt (Karolinska Institutet, Stockholm). Epinephrine was purchased from Parke-Davis and was used within 15 min of opening the vial. Highly purified glutaraldehyde was obtained from Ladd Research Industries, Inc., Burlington, Vermont. Lubrol was obtained from Imperial Chemical Industries, Providence, Rhode Island. The sources of all other reagents were specified previously (10–12).

#### *Methods*

Plasma membranes were prepared from livers of male and female Sprague-Dawley rats weighing 140 to 180 g according to the procedures devised by Neville (5). The gradient for Step 13[2] of the procedure was a 25-ml linear gradient from 26 to 1% sucrose prepared with a Beckman Gradient Former. These membranes are referred to below as "fully purified membranes." "Partially purified membranes" were prepared by

[1] The abbreviation used is: cyclic AMP, cyclic adenosine 3′,5′-monophosphate.

[2] The step numbers used here are those used by Neville (5) in his description of the liver membrane preparation.

doubling the amount of starting material and the volume of each of the steps, and omitting the final rate-zonal density gradient centrifugation (Steps 12 to 15). In some experiments, one-half of partially purified membrane pellet was carried through the final centrifugation and used as fully purified membranes. All membrane preparations were either used immediately or were stored immediately in liquid nitrogen as compact pellets (25,000 × *g*). For routine use, one or two batches of membranes were suspended in 1 mM $KHCO_3$ at a concentration of 8.0 to 18.0 mg of membrane protein per ml and distributed as 0.1- or 0.2-ml aliquots in individual glass tubes. These tubes were stored in liquid nitrogen and thawed individually as needed in order to avoid repeated freezing and thawing of the entire batch of membranes.

*Adenyl Cyclase Assay*—Adenyl cyclase activity was measured by the method of Krishna, Weiss, and Brodie (10) as described previously (11, 12). Unless specified otherwise, the following conditions were standard. The assay medium contained 3.2 mM ATP-$\alpha$-$^{32}$P (25 to 50 cpm per pmole), 5 mM $MgCl_2$, 1 mM EDTA, 25 mM Tris-HCl, pH 7.6, an ATP-regenerating system consisting of 20 mM phosphocreatine and 1 mg per ml of creatine phosphokinase (20 to 50 units per mg), and 10 to 50 μg of membrane protein in a volume of 50 μl. Hormones were diluted in the creatine phosphokinase solution. Incubations were initiated by the addition of the membranes and were continued for 10 min at 30°. Reactions were terminated by boiling for 3½ min.

Protein was measured by the Lowry procedure (13) using bovine serum albumin as standard.

*"Marker" Enzymes*—5'-Nucleotidase (EC 3.1.3.5) was measured by the method of Bodansky and Schwartz (14), glucose 6-phosphatase (EC 3.1.3.9) by the method of Swanson (15) with addition of 1 mM EDTA, alkaline phosphatase (EC 3.1.3.1) by the method of Heppel (16), and acid phosphatase (EC 3.1.3.2) by the same method using 100 mM sodium acetate buffer, pH 5.3. These four enzymes were assayed in 0.1 ml of appropriate medium and incubations were at 30°. Incubation time varied between 10 and 90 min depending on enzyme fraction and activi *y* measured. Activities were proportional to enzyme concentration. Reactions were stopped by addition of 0.025 ml 50% trichloracetic acid. The precipitate was removed by centrifugation, and the liberated $P_i$ was determined on 0.05-ml aliquots by the method of Fiske and SubbaRow (17). Succinate-cytochrome *c* reductase was measured by the method described by Fleischer and Fleischer (18).

*Electron Microscopy*—Liver membrane suspensions were centrifuged in 0.4-ml centrifuge tubes for 15 min at 25,000 × *g*. The pellets contained about 1 mg of protein and measured about 0.5 × 0.2 mm. The pellets were fixed at 0° for 30 min in 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The fixative was removed and the pellet rinsed for 1 hour at 0° with buffer. Post-fixation was then performed for 2 hours at 0° in 2% osmium tetroxide in phosphate buffer followed by dehydration in cold acetone. The pellets were then embedded in epoxy resin (19) and oriented so that complete top to bottom sections could be obtained. Sectioning was performed with a Reichert Om U 2 microtome. Sections were stained in a lead hydroxide solution (20). Electron micrographs were obtained with a Philips EM 300 electron microscope employing a 40 μ objective aperture and an accelerating voltage of 80 kv.

*Expression of Results and Terminology*—In all figures and tables "adenyl cyclase activity" refers to nanomoles of cyclic AMP formed in 10 min per mg of membrane protein. Basal activity is the activity measured in the absence of glucagon or fluoride ion. Glucagon-, epinephrine-, and fluoride-stimulated activities are activities measured in the presence of these compounds.

## RESULTS

Membranes prepared by the complete and the modified procedure of Neville (5) are referred to below as fully and partially purified membranes, respectively. The yield of fully purified membranes is 0.5 to 0.8 mg of membrane protein per g, wet weight, of liver. The yield of partially purified membranes is 1.0 to 2.0 mg of membrane protein per g, wet weight, of liver.

The purity of membrane preparations was checked by electron microscopy and by assay of marker enzymes. The fully purified plasma membrane preparations are composed mainly of membrane sheets (Fig. 1*A*) usually arranged in pairs joined by various kinds of cell junctions (Fig. 1, *B* and *C*). In addition, a relatively small number of vesicles are present (Fig. 1*A*). No other organelles were recognized. The paired membrane sheets with typical intercellular junctions and the bile canaliculus structure (not shown; see Reference 21) are unequivocally identified as originating from the plasma membrane of hepatic parenchymal cells (21). The origin of the vesicles is less certain. However, the suggestion has been made that they represent fragments of the "blood front" plasma membrane (22), the region of the plasma membrane not immediately adjacent to another parenchymal cell.

The partially purified membranes differ from the fully purified membranes mainly in the larger number of vesicles relative to sheets (Fig. 1*D*). In addition, occasional mitochondria and a significant number of small, densely stained, unidentified contaminants (Fig. 1*D*) were seen.

The marker enzyme data, summarized in Table I, demonstrates a substantial increase in specific activity of 5'-nucleotidase, a plasma membrane marker (22), in both the partially and fully purified membranes relative to the crude homogenate. The glucose 6-phosphatase and succinate-cytochrome *c* reductase activities indicate significant microsomal and mitochondrial contamination of the partially purified membrane preparation and that this contamination is substantially reduced in the fully purified membranes.

*Purification and Yield of Adenyl Cyclase in Membrane Preparations*—Table II summarizes the purification and yield of basal, glucagon-, epinephrine-, and fluoride-stimulated adenyl cyclase activities in partially and fully purified plasma membranes compared to a crude homogenate of liver. The glucagon-sensitive adenyl cyclase activity is obtained in greatest yield and specific activity in the partially purified membranes; a 25-fold purification is obtained in this material. The fully purified membranes have both a lower yield and lower specific activity of glucagon-sensitive activity. Both preparations have several-fold higher specific activity than a 1500 × *g* pellet of the liver homogenate. Epinephrine-sensitive adenyl cyclase is present in both membrane preparations at much lower activity than glucagon-sensitive activity but is also substantially enriched in comparison to the homogenate activity.

*Stimulation of Adenyl Cyclase Activity in Membrane Preparations by Hormones and Fluoride Ion*—Glucagon produces by far the largest stimulation of the liver membrane adenyl cyclase activity of any agent which we have tested; the stimulation

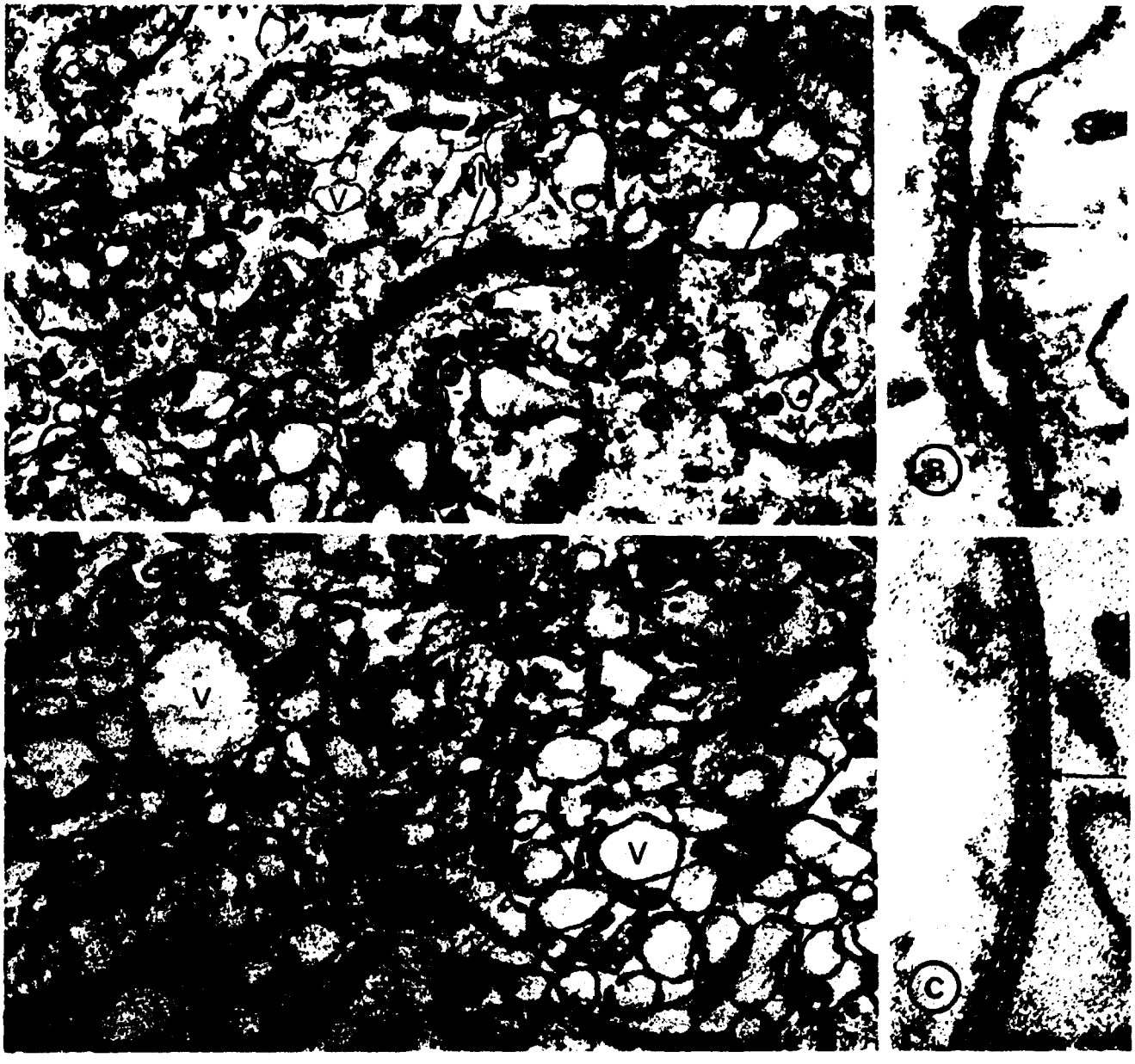


FIG. 1. Electron micrographs of membrane preparations. *PMS*, plasma membrane sheets; *V*, vesicle. *A*, fully purified membranes. Magnification × 30,000. *B*, higher magnification showing junctions between two membrane sheets and typical unit membrane structure (*arrow*). Magnification × 150,000. *C*, detail of a tight junction showing two membrane sheets with fused outer leaflets (*arrow*). Magnification × 300,000. *D*, partially purified membranes. *Arrow* indicates unidentified densely staining contaminant. Magnification × 30,000.

produced by glucagon is from 10 to 30 times the basal activity. The activities reported here are greater than those in our previous communication (4) primarily because of the addition of EDTA to the assay medium. The effect of EDTA is described below.

Epinephrine produces a small stimulation of the rat liver plasma membrane adenyl cyclase activity (Table III), an effect which we failed to observe in the preliminary experiments reported earlier (4). However, the epinephrine stimulation is only 1.5 to 2.5 times the basal activity or less than 10% of the stimulation produced by glucagon.

A variety of other polypeptide hormones tested, secretin, adrenocorticotropin, thyrotropin, luteinizing hormone, vasopressin, and parathyroid hormone, failed to stimulate the liver membrane adenyl cyclase system. The failure of secretin to stimulate is of particular interest since the primary structures of glucagon and secretin are similar (23). Insulin at a concentration of 10 munits per ml failed to change the basal or glucagon-stimulated adenyl cyclase activities.

The liver membrane adenyl cyclase system is sensitive to low concentrations of glucagon. As shown in Fig. 2, adenyl cyclase activity is a function of glucagon concentration over a range of $10^{-10}$ to $10^{-7}$ M with half-maximal stimulation occurring at

about $4 \times 10^{-9}$ M. The maximal activity obtained with glucagon is greater in the partially purified than in the fully purified membranes. The other parameters of the dose-response relationship of adenyl cyclase activity to glucagon concentration are identical in the two preparations.

Fluoride ion stimulates adenyl cyclase activity in the liver membrane preparations (Table II). The fluoride concentration dependence of this effect is similar in the partially and fully purified membranes (Fig. 3). The effect of fluoride ion will be treated more fully in a separate report (6).

The liver membrane preparations may be stored in liquid nitrogen for at least 3 months without loss of glucagon- or fluoride-stimulated adenyl cyclase activities.

*Assay Conditions for Adenyl Cyclase Activity*—Fig. 4 presents the time course of the glucagon-stimulated adenyl cyclase reaction with varying amounts of liver membrane protein assayed in media with and without ATP-regenerating system. It is apparent that under the standard assay conditions specified under "Methods," *i.e.* 10 to 50 μg of membrane protein incubated for 10 min in a medium containing ATP-regenerating system, the glucagon-stimulated adenyl cyclase activity is proportional to both time of incubation and to membrane concentration. This is also true for the fluoride-stimulated activity (data not shown). Without the ATP-regenerating system, the reaction is linear only for relatively short incubation times and at low membrane concentrations. The liver plasma membrane preparation contains a potent ATPase (24). Even with the ATP-regenerating system, the proportionality to time and membrane concentration is lost at membrane protein concentrations above 50 μg per assay (50 μl). The reason or reasons for this nonproportionality at high membrane concentrations is unknown, but several factors may contribute including aggregation of membranes, destruction of glucagon, inadequacy of the ATP-regenerating system, or production of an inhibitor.

The pH of the standard assay medium, 7.6, is the optimum for glucagon- and fluoride-stimulated activities in three buffer systems. Although greater enzyme activities were measured at 37° and 42°, the time courses were nonlinear at these temperatures; therefore, 30° was chosen as the standard incubation temperature. Addition of 1 mM EDTA to the assay medium

TABLE I

*Activities of marker enzymes in liver homogenates and in partially and fully purified membranes*

Rat livers were ground in a Dounce homogenizer as described by Neville (5). An aliquot of this crude homogenate was reground in a ground glass homogenizer to facilitate pipetting. The remainder of the homogenate was used to prepare partially and fully purified membranes as described under "Methods."

| Enzyme | Specific activity in | | |
|---|---|---|---|
| | Crude homogenate | Partially purified plasma membranes | Fully purified plasma membranes |
| | *μmole/min/mg protein* | | |
| 5'-Nucleotidase | 0.032 | 0.535 | 0.632 |
| Glucose 6-phosphatase | 0.038 | 0.021 | 0.012 |
| Alkaline phosphatase | 0.0041 | 0.015 | 0.016 |
| Acid phosphatase | 0.019 | 0.008 | 0.006 |
| Succinate-cytochrome *c* reductase | 0.025 | 0.028 | 0.003 |

TABLE III

*Hormone specificity of adenyl cyclase in liver membranes*

Partially and fully purified plasma membranes were assayed for adenyl cyclase activity in media containing no stimulant, epinephrine, secretin, glucagon, or 10 mM NaF. Hormones, when present, were at 20 μg per ml. Activities are expressed as the mean ± half the range of triplicate determinations.

| Addition | Adenyl cyclase activity | |
|---|---|---|
| | Partially purified plasma membranes | Fully purified plasma membranes |
| | *nmoles/10 min/mg protein* | |
| None | 0.30 ± 0.06 | 0.16 ± 0.01 |
| Epinephrine | 0.42 ± 0.02 | 0.28 ± 0.04 |
| Secretin | 0.34 ± 0.02 | 0.23 ± 0.04 |
| Glucagon | 3.33 ± 0.02 | 2.76 ± 0.02 |
| NaF | 1.92 ± 0.03 | 1.61 ± 0.06 |

TABLE II

*Purification of adenyl cyclase*

Adenyl cyclase activities of the crude homogenate and the partially and fully purified membrane preparations described in Table I were measured under standard assay conditions except that 1 mM cyclic AMP and 10 mM theophylline were added to the medium. When present, glucagon and epinephrine concentrations were 20 μg per ml and NaF was 10 mM. Adenyl cyclase activity is expressed as nanomoles of cyclic AMP formed in 10 min per mg of protein.

| Preparation | Total protein | Basal adenyl cyclase activity | Increase[a] in adenyl cyclase activity[b] due to addition of | | | Purification | | | Yield | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Glucagon | Epinephrine | NaF | Glucagon | Epinephrine | NaF | Glucagon | Epinephrine | NaF |
| | *mg* | | | | | | | | % | | |
| Homogenate | 17,000 | 0.034 | 0.244 | 0.036 | 0.194 | 1 | 1 | 1 | 100 | 100 | 100 |
| Partially purified plasma membranes | 117 | 0.207 | 6.136 | 0.506 | 2.558 | 25 | 14 | 13 | 14 | 3.5 | 8.0 |
| Fully purified plasma membranes | 21 | 0.172 | 4.166 | 0.273 | 2.651 | 17 | 9 | 13 | 1.0 | 0.2 | 1.0 |

[a] Basal activity subtracted.

[b] The glucagon-stimulated activities in this experiment are somewhat greater than those in other experiments described here possibly because it was performed several months later during which time the supplier of the ATP-α-$^{32}$P made a significant change in the preparative procedure.

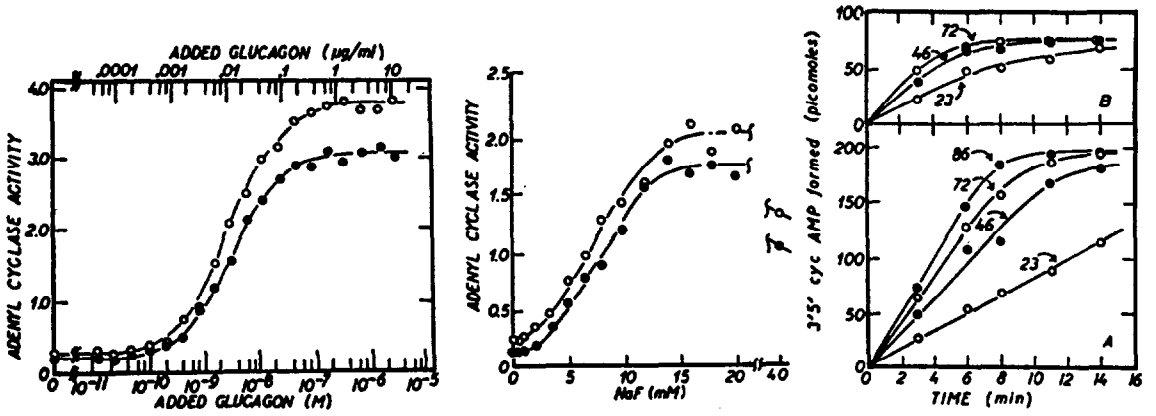


FIG. 2 (*left*). Effect of glucagon concentration on adenyl cyclase activity in partially purified (○) and fully purified (●) plasma membranes.

FIG. 3 (*center*). Effect of fluoride ion concentration on adenyl cyclase activity in partially purified (○) and fully purified plasma membranes (●).

FIG. 4 (*right*). Time course of the glucagon-stimulated adenyl cyclase reaction. Partially purified membranes were incubated for varying times in adenyl cyclase assay medium containing 10 μg per ml of glucagon prepared with (*A*) and without (*B*) the ATP-regenerating system. *Numbers on curves* indicate micrograms of membrane protein used for each study. Assay conditions were otherwise the same as described under "Methods." ○ and ●, serve only to distinguish adjacent curves.

caused a doubling of the glucagon-stimulated activity and lesser increases in the basal and fluoride-stimulated activities (Table IV).

The effects of varying ATP and magnesium concentrations on adenyl cyclase activity are shown in Figs. 5 and 6, respectively. Since the substrate concentration cannot be optimized for both glucagon- and fluoride-stimulated activities, 3.2 mM was chosen arbitrarily for standard assay conditions because it gives near optimal activities for both stimulants. In the presence of 3.2 mM ATP, 5 mM $Mg^{++}$ gives optimum basal, glucagon-, and fluoride-stimulated activities. Manganous ion may be substituted for magnesium (6) but calcium cannot and, at concentrations above $10^{-4}$ M, is inhibitory in the presence of magnesium.

A cyclic phosphodiesterase inhibitor, theophylline or caffeine, was not included in the standard assay medium because of our earlier finding (4) that the cyclic phosphodiesterase activity of the liver membrane preparation is so low as not to interfere with the adenyl cyclase assay under routine assay conditions with both partially and fully purified membranes.

*"Solubilization" Experiments*—A variety of methods were employed to alter the membranes in such a way that the adenyl cyclase activity would remain in the supernatant for at least 1 hour in a centrifugal field exceeding 100,000 × *g*. These included sonic oscillation, succinylation, and exposure to high salt concentrations, urea, and detergents. All of these treatments either abolished the adenyl cyclase activity or failed to solubilize the enzyme. Using the Lubrol procedure devised by Levey (25), approximately 15% of the fluoride-stimulated activity was obtained in a high speed supernatant. However, this activity was insensitive to glucagon.

*Effects of Glutaraldehyde, Sulfhydryl Reagents, and Urea*—As shown in Table V, glutaraldehyde treatment completely inactivates the adenyl cyclase system of the rat liver membrane preparation. Adenyl cyclase activity is also inhibited by treatment of the membranes with *N*-ethylmaleimide and *p*-chloromercuribenzoate, agents which react with sulfhydryl groups (26). In other experiments, the extent of inhibition by sulf-

TABLE IV

*Effects of EDTA on adenyl cyclase activity*

Partially purified membranes were incubated in standard assay medium with or without 1 mM EDTA. When present, glucagon was 10 μg per ml and NaF was 10 mM.

| Addition | Adenyl cyclase activity | |
|---|---|---|
| | −EDTA | +EDTA |
| None | 0.17 | 0.30 |
| Glucagon | 1.65 | 3.33 |
| NaF | 1.59 | 1.92 |

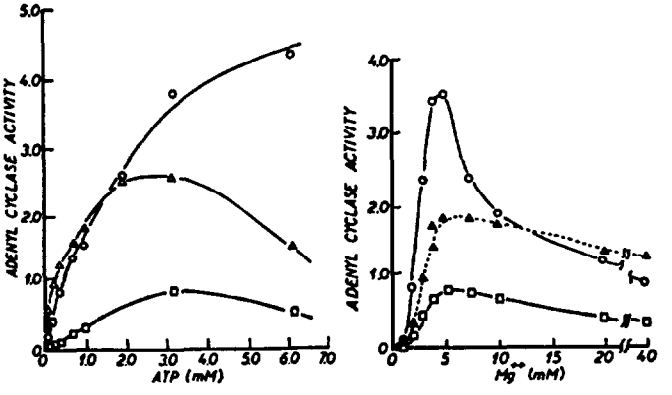


FIG. 5 (*left*). Effect of varying ATP concentration on basal, glucagon-, and fluoride-stimulated adenyl cyclase activities. Partially purified membranes were incubated in media containing no stimulant (□), 10 μg per ml of glucagon (○), 10 mM NaF (△), and varying concentrations of ATP. Other conditions were as described under "Methods."

FIG. 6 (*right*). Effect of varying $Mg^{++}$ concentrations on basal, glucagon-, and fluoride-stimulated adenyl cyclase activities. Partially purified membranes were incubated in media containing no stimulant (□), 10 μg per ml of glucagon (○), 10 mM NaF (▲), and varying concentrations of $MgCl_2$. Other conditions were as described under "Methods."

TABLE V

*Effects of glutaraldehyde and sulfhydryl reagents on adenyl cyclase activity*

For treatment with glutaraldehyde, partially purified membranes, 28 μg of membrane protein, were incubated in 0.02 ml of a medium containing 50 mM cacodylate-nitrate buffer, pH 7.4, and 4.8% sucrose with or without 1% glutaraldehyde. After 10 min at 30°, adenyl cyclase assay reagents were added in a volume of 0.03 ml and adenyl cyclase activity was determined as described under "Methods." For treatment with sulfhydryl reagents, fully purified membranes, 18 μg of membrane protein, were incubated in 0.02 ml of a medium containing 25 mM Tris-HCl, pH 7.4, and no addition, 1 mM *N*-ethylmaleimide, or 0.05 mM *p*-chloromercuribenzoate. After 15 min at 22°, adenyl cyclase assay reagents were added in a volume of 0.03 ml. EDTA was omitted from the standard assay medium, and 2-mercaptoethanol was included at a final concentration of 1 mM. When present, glucagon was 10 μg per ml and NaF was 10 mM.

| Treatment | Adenyl cyclase activity in the presence of | | |
|---|---|---|---|
| | Basal | Glucagon | NaF |
| Control | 0.78 | 4.86 | 2.96 |
| Glutaraldehyde | 0.02 | 0.01 | 0.04 |
| Control | 0.08 | 2.41 | 3.21 |
| *N*-Ethylmaleimide | 0.03 | 1.09 | 2.20 |
| *p*-Chloromercuribenzoate | 0.00 | 1.26 | 1.49 |

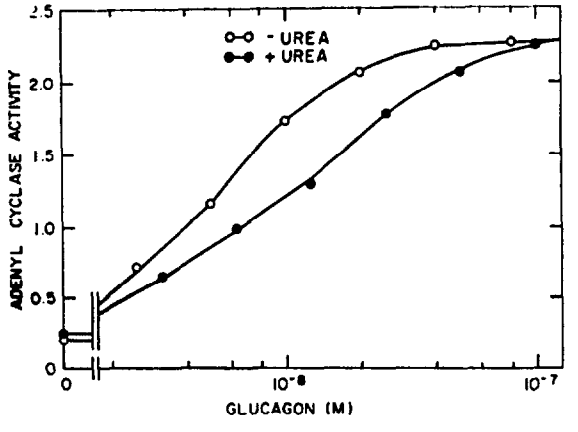


FIG. 7. Effect of the addition of 0.4 M urea to the standard adenyl cyclase assay medium on the dose-response relationship between glucagon and adenyl cyclase activity.

hydryl reagents was found to be nearly complete with longer incubation times, and addition of 2-mercaptoethanol or dithiothreitol was found to block the effect of these agents.

Incubation of the membranes in 2 M urea for 10 min at 30° reduces both the glucagon- and fluoride-stimulated activities by 60 to 80%. Addition of 0.4 M urea to the adenyl cyclase assay medium decreases the apparent affinity of the system for glucagon (Fig. 7). Other effects of urea on the adenyl cyclase system will be described in a later paper (7).

## DISCUSSION

Adenyl cyclase activity is widely distributed in mammalian tissues (1, 3) and is controlled by a variety of polypeptide hormones and catecholamines (3). Despite the central position of the enzyme system in hormone action, little is known of the mechanism by which hormones control its activity. It is membrane-bound and loses its response to hormones when attempts are made to solubilize and purify its components. For this reason, we have studied adenyl cyclase systems in intact membranes and have employed indirect means of characterizing the components (12, 27, 28).

Previous studies of the adenyl cyclase system in fat cell ghosts indicated that at least five different hormones stimulate the activity of a single catalytic component through discrete receptors (27, 28), and that the affinity of an allosteric site for magnesium may be involved in the mechanism of hormonal stimulation (12). Further study of the fat cell ghost system was hampered by the small amounts of this material that can practically be obtained and by its relative instability.

The finding of a glucagon-sensitive adenyl cyclase in the plasma membrane preparation devised by Neville was initially of interest because it added further evidence for the cellular location of adenyl cyclase in plasma membranes (4, 29–31). In addition, it can be prepared in large quantities and is stable for long periods of time in the frozen state. Furthermore, liver plasma membrane preparations have been characterized more extensively (5, 22, 32, 33) than any other source of plasma membranes containing hormonally sensitive adenyl cyclase.

Fully purified liver plasma membranes consist of paired membrane sheets with junctions and the bile canaliculus structure which unequivocally identify the origin of these sheets as the plasma membrane of hepatic parenchymal cells. Vesicles are also present which, according to Benedetti and Emmelot (22), may be derived from the plasma membrane because they are isopycnic with the membrane sheets and because preparations containing vesicles have low specific activities for enzyme and chemical markers of other organelles. This suggestion is supported by the report (33) that purified liver plasma membranes contain undetectable amounts of a unique glycolipid found in other types of liver membranes. Our finding that the fully and partially purified membranes, the latter being rich in vesicles, have the same specific activity of 5′-nucleotidase, a plasma membrane marker (34), adds to the evidence that the vesicles are derived from plasma membrane.

Adenyl cyclase activity is considerably enriched, relative to liver homogenates, in both partially and fully purified membranes. The similarity in the two preparations of the dose response curves for glucagon and fluoride ion suggests that omission of the final purification step does not introduce adenyl cyclase with different properties from that in the fully purified membranes. The glucose 6-phosphatase and succinate-cytochrome *c* reductase activities indicate a considerable contamination of the partially purified membranes with other organelles, but because of the much higher yield of adenyl cyclase activity we have considered the partially purified membranes preferable to the fully purified membranes for our studies.

It should be emphasized that the present studies do not indicate, particularly with the partially purified membrane preparation, that adenyl cyclase is present exclusively in plasma membranes of parenchymal cells. There is histochemical evidence (35) that glucagon- and epinephrine-sensitive adenyl cyclase systems are present in plasma membranes of both reticuloendothelial and parenchymal cells. It is possible that the higher specific activity of the glucagon-stimulated adenyl cyclase in

partially purified membranes represents contributions of both parenchymal and reticuloendothelial cells. Unfortunately, quantitative assessment of the contributions of glucagon-sensitive adenyl cyclase from the two cell types cannot be made from the histochemical study since glutaraldehyde, used as fixative, inactivates adenyl cyclase in isolated membranes and causes substantial loss of activity in blocks or sections of liver used for the histochemical studies (35). Studies of the hormone responsiveness of isolated parenchymal and reticuloendothelial cells may provide a definitive answer to this question.

Glucagon, of the several hormones and other agents tested, produced the greatest stimulation of adenyl cyclase activity in the liver membrane preparations. In contrast to our previous report (4), epinephrine also produced a stimulation of adenyl cyclase activity although to a much lesser degree than did glucagon. Bitensky *et al.* (36, 37) have provided evidence for two different adenyl cyclase systems in liver homogenates, one sensitive to glucagon and the other to epinephrine. Our finding that glucagon- and epinephrine-sensitive activities are not increased by the same factor in partially and fully purified membranes compared to the homogenate is consistent with the hypothesis that there are two adenyl cyclase systems present in liver.

Marinetti *et al.* (38, 39) have reported recently a much greater epinephrine stimulation of adenyl cyclase in rat liver membranes prepared in a different manner than in this study. They also reported several other observations which are opposed to our findings in the liver membrane preparation and the findings of other investigators (3) in different tissues. For example, they report inhibition by fluoride, activation by calcium ion, inhibition by insulin, and activation of adenyl cyclase by *p*-chloromercuribenzoate. Finally, their preparation appears to be 100- to 1000-fold less sensitive to glucagon than our preparation.

Fluoride ion stimulates the liver plasma membrane adenyl cyclase system as it does all other mammalian systems (3). However, under our standard assay conditions, the activity obtained with a maximally stimulating concentration of glucagon is about two times that obtained with a maximally stimulating concentration of fluoride ion. Fluoride-stimulated activity is not exceeded by hormonal stimulation in several other systems (3, 12, 40, 41) and the suggestion has been made that an appropriate concentration of fluoride ion produces maximal expression of enzyme activity (1, 41). Inclusion of 1 mM EDTA into the incubation medium in the present study enhanced the response of liver adenyl cyclase to glucagon. In the following report (6) it will be seen that a number of agents selectively alter the response of liver adenyl cyclase to fluoride ion and glucagon.

The adenyl cyclase assay conditions and method of measurement have been adequately described in publications from this and other laboratories (4, 10–12, 25, 38, 41) and do not require extensive comment. However, it is important to note that under our standard assay conditions, the measured activity is proportional to time of incubation and to membrane concentration. The liver plasma membrane preparation contains both a potent ATPase (24) and a system which rapidly inactivates glucagon in the assay medium (7). Consequently, any significant change in the composition of the assay medium requires at least that the linearity of enzyme activity with time be checked.

Mammalian adenyl cyclase systems are highly complex and probably multimolecular. An understanding, in molecular terms, of the mechanism or mechanisms by which polypeptide hormones control these systems depends upon identification, characterization, and isolation of the components of which such systems are made. The liver plasma membrane preparation, with its glucagon-sensitive adenyl cyclase system, provides a useful starting material for such studies.

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